

Claims

What is claimed is:

5 1. A microbial trypsin variant having chymotrypsin-like activity, comprising one or more modifications selected from the group consisting of:

(a) a substitution at one or more positions corresponding to positions 144, 193, 198, 201, 218, 223, 227, 228, 229, 230, and 231 of amino acids 25 to 248 of SEQ ID NO: 2;

10 (b) a deletion at one or more positions corresponding to positions 192, 197, and 226 of amino acids 25 to 248 of SEQ ID NO: 2; and

(c) an insertion between positions corresponding to positions 224 and 225 of amino acids 25 to 248 of SEQ ID NO: 2;

wherein the microbial trypsin is (a) a polypeptide having an amino acid sequence which has at least 70% identity to amino acids 25 to 248 of SEQ ID NO: 2; or (b) a polypeptide encoded by a nucleotide
15 sequence which hybridizes under at least low stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or its complementary strand, wherein the variant has chymotrypsin-like activity and has an amino acid sequence that has at least 70% identity to the amino acid sequence of the microbial trypsin.

20 2. The variant of claim 1, wherein the variant comprises a substitution at position 144.

3. The variant of claim 2, wherein the substitution is Thr.

4. The variant of claim 1, which comprises a substitution at position 193.

25 5. The variant of claim 4, wherein the substitution is Ala.

6. The variant of claim 1, which comprises a substitution at position 198.

30 7. The variant of claim 6, wherein the substitution is Ser.

8. The variant of claim 1, which comprises a substitution at position 201.

9. The variant of claim 8, wherein the substitution is Met.

10. The variant of claim 1, which comprises a substitution at position 218.
11. The variant of claim 10, wherein the substitution is Ile.
- 5 12. The variant of claim 1, which comprises a substitution at position 223.
13. The variant of claim 12, wherein the substitution is Ser.
14. The variant of claim 1, which comprises a substitution at position 227.
- 10 15. The variant of claim 14, wherein the substitution is Ser.
16. The variant of claim 1, which comprises a substitution at position 228.
- 15 17. The variant of claim 16, wherein the substitution is Thr.
18. The variant of claim 1, which comprises a substitution at position 229.
19. The variant of claim 18, wherein the substitution is Ser.
- 20 20. The variant of claim 1, which comprises a substitution at position 230.
21. The variant of claim 20, wherein the substitution is Thr.
- 25 22. The variant of claim 1, which comprises a substitution at position 231.
23. The variant of claim 22, wherein the substitution is Pro.
24. The variant of claim 1, which comprises one or more substitutions selected from the group
30 consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and
S231P.
25. The variant of claim 1, which comprises the substitutions V144T + S193A + D198S + Q201M
+ A218I + N223S + R227S + P228T + N229S + Y230T + S231P.
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26. The variant of claim 1, which comprises a deletion at position 192.

27. The variant of claim 26, wherein the deletion is Val.

5 28. The variant of claim 1, which comprises a deletion at position 197.

29. The variant of claim 28, wherein the deletion is Lys.

30. The variant of claim 1, which comprises a deletion at position 226.

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31. The variant of claim 30, wherein the deletion is Ala.

32. The variant of claim 1, which comprises one or more substitutions selected from the group consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and
15 S231P; and one or more deletions selected from the group consisting of V192*, K197*, and A226*.

33. The variant of claim 1, which comprises the substitutions V144T + S193A + D198S + Q201M + A218I + N223S + R227S + P228T + N229S + Y230T + S231P, and the deletions V192* + K197* + A226*.

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34. The variant of claim 1, which comprises an insertion between positions 224 and 225.

35. The variant of claim 34, wherein the insertion is Thr.

25 36. The variant of claim 1, which comprises one or more substitutions selected from the group consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and S231P; one or more deletions selected from the group consisting of V192*, K197*, and A226*; and the insertion G224GT of amino acids 25 to 248 of SEQ ID NO: 2.

30 37. The variant of claim 1, which comprises the substitutions V144T + S193A + D198S + Q201M + A218I + N223S + R227S + P228T + N229S + Y230T + S231P, and the deletions V192* + K197* + A226*, and the insertion G224GT of amino acids 25 to 248 of SEQ ID NO: 2.

35 38. The variant of claim 1, wherein the microbial trypsin has an amino acid sequence which has at least 70% identity with amino acids 25 to 248 of SEQ ID NO: 2.

39. The variant of claim 38, wherein the microbial trypsin has an amino acid sequence which has at least 75% identity with amino acids 25 to 248 of SEQ ID NO: 2.

5 40. The variant of claim 39, wherein the microbial trypsin has an amino acid sequence which has at least 80% identity with amino acids 25 to 248 of SEQ ID NO: 2.

41. The variant of claim 40, wherein the microbial trypsin has an amino acid sequence which has at least 85% identity with amino acids 25 to 248 of SEQ ID NO: 2.

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42. The variant of claim 41, wherein the microbial trypsin has an amino acid sequence which has at least 90% identity with amino acids 25 to 248 of SEQ ID NO: 2.

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43. The variant of claim 42, wherein the microbial trypsin has an amino acid sequence which has at least 95% identity with amino acids 25 to 248 of SEQ ID NO: 2.

44. The variant of claim 1, wherein the microbial trypsin has the amino acid sequence of amino acids 25 to 248 of SEQ ID NO: 2.

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45. The variant of claim 1, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under low stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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46. The variant of claim 45, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under medium stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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47. The variant of claim 46, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under medium-high stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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48. The variant of claim 47, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under high stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

49. The variant of claim 1, wherein the microbial trypsin is a wild-type microbial trypsin.

50. The variant of claim 1, wherein the microbial trypsin is an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Botryosphaeria*, *Chaetomidium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*,
5 *Cryphonectria*, *Exidia*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*,
Leptosphaeria, *Melanocarpus*, *Neurospora*, *Penicillium*, *Phanerochaete*, *Poitrasia*,
Pseudotriconympha, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Trichoderma*,
Trichophaea, *Verticillium*, *Volvariella*, and *Xylaria* trypsin.

10 51. The variant of claim 50, wherein the *Fusarium* trypsin is a *Fusarium oxysporum* trypsin.

52. The variant of claim 1, wherein the total number of substitutions is 11, more preferably 10,
even more preferably 9, even more preferably 8, even more preferably 7, even more preferably 6,
even more preferably 5, even more preferably 4, even more preferably 3, even more preferably 2,
15 and most preferably 1.

53. The variant of claim 1, wherein the total number of deletions is 3, more preferably 2, and
most preferably 1.

20 54. The variant of claim 1, which has an amino acid sequence with at least 70% identity to the
amino acid sequence of the microbial trypsin.

55. The variant of claim 54, which has an amino acid sequence with at least 75% identity to the
amino acid sequence of the microbial trypsin.

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56. The variant of claim 55, which has an amino acid sequence with at least 80% identity to the
amino acid sequence of the microbial trypsin.

57. The variant of claim 56, which has an amino acid sequence with at least 85% identity to the
30 amino acid sequence of the microbial trypsin.

58. The variant of claim 57, which has an amino acid sequence with at least 90% identity to the
amino acid sequence of the microbial trypsin.

35 59. The variant of claim 58, which has an amino acid sequence with at least 95% identity to the

amino acid sequence of the microbial trypsin.

60. The variant of claim 1, which is encoded by the nucleotide sequence contained in pEJG66.1XLGOLD which is contained in *E. coli* NRRL B-30627.

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61. The variant of claim 1, which is in the form of a precursor comprising amino acids 1 to 24 of SEQ ID NO: 2 as the prepro region, or a portion thereof, linked in translation reading frame with the amino terminus of the trypsin variant.

10 62. An isolated nucleotide sequence encoding the variant of claim 1.

63. An expression vector comprising the nucleotide sequence of claim 62.

64. A host cell comprising the expression vector of claim 63.

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65. The host cell of claim 64, which is a bacterium, fungus, insect, mammalian, or plant cell.

66. The host cell of claim 65, wherein the fungus is a yeast.

20 67. The host cell of claim 65, wherein the fungus is a filamentous fungus.

68. A method for obtaining a variant of a microbial trypsin, comprising:

(a) introducing one or more modifications selected from the group consisting of:

25 (1) a substitution at one or more positions corresponding to positions 144, 193, 198, 201, 218, 223, 227, 228, 229, 230, and 231 of amino acids 25 to 248 of SEQ ID NO: 2,

(2) a deletion at one or more positions corresponding to positions 192, 197, and 226 of amino acids 25 to 248 of SEQ ID NO: 2; and

(3) an insertion between positions corresponding to positions 224 and 225 of amino acids 25 to 248 of SEQ ID NO: 2;

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wherein the microbial trypsin is (a) a polypeptide having an amino acid sequence which has at least 70% identity to amino acids 25 to 248 of SEQ ID NO: 2; or (b) a polypeptide encoded by a nucleotide sequence which hybridizes under low stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or its complementary strand, wherein the variant has microbial trypsin activity and has an amino acid sequence that has at least 70% identity to the amino acid sequence of the microbial trypsin; and

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(b) recovering the variant having chymotrypsin-like activity.

69. A method for producing a microbial trypsin variant, comprising:

(a) cultivating a host cell under conditions suitable for the expression of the variant,
5 wherein the host cell comprises a nucleotide sequence encoding the variant comprising one or more modifications selected from the group consisting of:

(a) a substitution at one or more positions corresponding to positions 144, 193, 198, 201, 218, 223, 227, 228, 229, 230, and 231 of amino acids 25 to 248 of SEQ ID NO: 2,

(b) a deletion at one or more positions corresponding to positions 192, 197, and 226 of
10 amino acids 25 to 248 of SEQ ID NO: 2; and

(c) an insertion between positions corresponding to positions 224 and 225 of amino acids 25 to 248 of SEQ ID NO: 2;

wherein the microbial trypsin is (a) a polypeptide having an amino acid sequence which has at least 70% identity to amino acids 25 to 248 of SEQ ID NO: 2; or (b) a polypeptide encoded by a
15 nucleotide sequence which hybridizes under low stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or its complementary strand, wherein the variant has microbial trypsin activity and has an amino acid sequence that has at least 70% identity to the amino acid sequence of the microbial trypsin; and

(b) recovering the variant from the cultivation medium.

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70. The method of claim 69, wherein the variant comprises a substitution at position 144.

71. The method of claim 70, wherein the substitution is Thr.

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72. The method of claim 69, wherein the variant comprises a substitution at position 193.

73. The method of claim 72, wherein the substitution is Ala.

74. The method of claim 69, wherein the variant comprises a substitution at position 198.

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75. The method of claim 74, wherein the substitution is Ser.

76. The method of claim 69, wherein the variant comprises a substitution at position 201.

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77. The method of claim 76, wherein the substitution is Met.

78. The method of claim 69, wherein the variant comprises a substitution at position 218.
79. The method of claim 78, wherein the substitution is Ile.
- 5 80. The method of claim 69, wherein the variant comprises a substitution at position 223.
81. The method of claim 80, wherein the substitution is Ser.
- 10 82. The method of claim 69, wherein the variant comprises a substitution at position 227.
83. The method of claim 82, wherein the substitution is Ser.
84. The method of claim 69, wherein the variant comprises a substitution at position 228.
- 15 85. The method of claim 84, wherein the substitution is Thr.
86. The method of claim 69, wherein the variant comprises a substitution at position 229.
- 20 87. The method of claim 86, wherein the substitution is Ser.
88. The method of claim 69, wherein the variant comprises a substitution at position 230.
89. The method of claim 88, wherein the substitution is Thr.
- 25 90. The method of claim 69, wherein the variant comprises a substitution at position 231.
91. The method of claim 90, wherein the substitution is Pro.
- 30 92. The method of claim 69, wherein the variant comprises one or more substitutions selected from the group consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and S231P.
- 35 93. The method of claim 69, wherein the variant comprises the substitutions V144T + S193A + D198S + Q201M + A218I + N223S + R227S + P228T + N229S + Y230T + S231P.

94. The method of claim 69, wherein the variant comprises a deletion at position 192.

95. The method of claim 94, wherein the deletion is Val.

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96. The method of claim 69, wherein the variant comprises a deletion at position 197.

97. The method of claim 96, wherein the deletion is Lys.

10 98. The method of claim 69, wherein the variant comprises a deletion at position 226.

99. The method of claim 98, wherein the deletion is Ala.

15 100. The method of claim 69, wherein the variant comprises one or more substitutions selected from the group consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and S231P; and one or more deletions selected from the group consisting of V192*, K197*, and A226*.

20 101. The method of claim 69, wherein the variant comprises the substitutions V144T + S193A + D198S + Q201M + A218I + N223S + R227S + P228T + N229S + Y230T + S231P, and the deletions V192* + K197* + A226*.

102. The method of claim 69, wherein the variant comprises an insertion between positions 224 and 225.

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103. The method of claim 102, wherein the insertion is Thr.

30 104. The method of claim 69, wherein the variant comprises one or more substitutions selected from the group consisting of V144T, S193A, D198S, Q201M, A218I, N223S, R227S, P228T, N229S, Y230T, and S231P; one or more deletions selected from the group consisting of V192*, K197*, and A226*; and the insertion G224GT of amino acids 25 to 248 of SEQ ID NO: 2.

35 105. The method of claim 69, wherein the variant comprises the substitutions V144T + S193A + D198S + Q201M + A218I + N223S + R227S + P228T + N229S + Y230T + S231P, the deletions V192* + K197* + A226*, and the insertion G224GT of amino acids 25 to 248 of SEQ ID NO: 2.

106. The method of claim 69, wherein the microbial trypsin has an amino acid sequence which has at least 70% identity with amino acids 25 to 248 of SEQ ID NO: 2.

5 107. The method of claim 106, wherein the microbial trypsin has an amino acid sequence which has at least 75% identity with amino acids 25 to 248 of SEQ ID NO: 2.

108. The method of claim 107, wherein the microbial trypsin has an amino acid sequence which has at least 80% identity with amino acids 25 to 248 of SEQ ID NO: 2.

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109. The method of claim 108, wherein the microbial trypsin has an amino acid sequence which has at least 85% identity with amino acids 25 to 248 of SEQ ID NO: 2.

110. The method of claim 109, wherein the microbial trypsin has an amino acid sequence which has at least 90% identity with amino acids 25 to 248 of SEQ ID NO: 2.

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111. The method of claim 110, wherein the microbial trypsin has an amino acid sequence which has at least 95% identity with amino acids 25 to 248 of SEQ ID NO: 2.

20 112. The method of claim 69, wherein the microbial trypsin has the amino acid sequence of amino acids 25 to 248 of SEQ ID NO: 2.

113. The method of claim 69, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under low stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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114. The method of claim 113, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under medium stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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115. The method of claim 114, wherein the microbial trypsin is encoded by a nucleotide sequence which hybridizes under medium-high stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

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116. The method of claim 115, wherein the microbial trypsin is encoded by a nucleotide sequence

which hybridizes under high stringency conditions with nucleotides 202 to 801 of SEQ ID NO: 1 or the nucleotide sequence of nucleotides 202 to 801 of SEQ ID NO: 1.

117. The method of claim 69, wherein the microbial trypsin is a wild-type microbial trypsin.

118. The method of claim 69, wherein the microbial trypsin is an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Botryosphaeria*, *Chaetomidium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Cryphonectria*, *Exidia*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Melanocarpus*, *Neurospora*, *Penicillium*, *Phanerochaete*, *Poitrasia*, *Pseudotriconympha*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, and *Xylaria* trypsin.

119. The method of claim 118, wherein the *Fusarium* trypsin is a *Fusarium oxysporum* microbial trypsin.

120. The method of claim 69, wherein the total number of substitutions is 11, more preferably 10, even more preferably 9, even more preferably 8, even more preferably 7, even more preferably 6, even more preferably 5, even more preferably 4, even more preferably 3, even more preferably 2, and most preferably 1.

121. The method of claim 69, wherein the total number of deletions is 3, more preferably 2, and most preferably 1.

122. The method of claim 69, which has an amino acid sequence with at least 70% identity to the amino acid sequence of the microbial trypsin.

123. The method of claim 122, which has an amino acid sequence with at least 75% identity to the amino acid sequence of the microbial trypsin.

124. The method of claim 123, which has an amino acid sequence with at least 80% identity to the amino acid sequence of the microbial trypsin.

125. The method of claim 124, which has an amino acid sequence with at least 85% identity to the amino acid sequence of the microbial trypsin.

126. The method of claims 125, which has an amino acid sequence with at least 90% identity to the amino acid sequence of the microbial trypsin.

127. The method of claim 126, which has an amino acid sequence with at least 95% identity to the amino acid sequence of the microbial trypsin.

128. The method of claim 69, wherein the variant is encoded by the nucleotide sequence contained in pEJG66.1XLGOLD which is contained in *E. coli* NRRL B-30627.

129. The method of claim 69, wherein the variant is in the form of a precursor comprising amino acids 1 to 24 of SEQ ID NO: 2 as the prepro region, or a portion thereof, linked in translation reading frame with the amino terminus of the trypsin variant.

130. A detergent composition comprising a variant of claim 1 and a surfactant.